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ANTIFUNGAL ACTIVITY OF VOLATILE COMPONENTS GENERATED BY ESSENTIAL OILS AGAINST THE GENUS *PENICILLIUM* ISOLATED FROM BAKERY PRODUCTS

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ABSTRACT

The aim of this study was evaluation of the antifungal activity of 5 essential oils (EOs). We concretely used thyme, clove, basil, jasmine and rosemary EOs by vapor contact against the fungal species, namely *Penicillium citrinum, P. chrysogenum, P. hordei, P. citreonigrum,* and *P. viridicatum* and their ability to affect production of mycotoxins. Each fungus was inoculated in the centre on Czapek Yeast Autolysate Agar (CYA) dishes. Dishes were tightly sealed with parafilm and incubated for fourteen days at 25 ± 1 °C (three replicates were used for each treatment). Volatile phase effect of 50 µl of the essential oils was found to inhibit on growth of *Penicillium* spp.. Complete growth inhibition of the isolates by EOs of thyme and clove was observed. The EO of basil had antifungal effect on growth of *P. citreonigrum* only after 3rd and 7th day of the incubation at concentration 100 % of EO, like a *P. viridicatum*, which was inhibited by basil EO (100 %) in comparison with control sets. Data was evaluated statistically by 95.0 % Tukey HSD test. In this study we also tested potentional effect of EOs to affect production of mycotoxins of tested *Penicillium* isolates which are potential toxigenic fungi. After 14 days of incubation with EOs (100 %) with control sets, they were screened for a production of mycotoxins by TLC chromatography. Results showed non affecting production of mycotoxins by tested EOs.
Conclusions indicate that volatile phase of combinations of thyme oil and clove oil showed good potential in the inhibition of growth of *Penicillium* spp. EOs should find a practical application in the inhibition of the fungal mycelial growth in some kind of the food.

Keywords: *Penicillium* spp., essential oils, vapor, antifungal activity, mycotoxins

INTRODUCTION

The present and growth of fungi in food may cause spoilage and result in a reduction in quality and quantity (**Baratta *et al.*, 1998**). Apart from their potentiality to cause yield losses and food decay, any of them represent very serious risk for consumers because of their production of dangerous secondary metabolites. This is also the topic of current concern related to safety of food production. Mold growth on bakery products during storage is a serious economic problem (**Seiler, 1998**). Mould spoilage of bakery products has been the subject of many studies and a number of species have been implicated. The mold frequently involved are *Penicillium, Aspergillus, Eurotium* and *Walemia* species (**Dantigny *et al.*, 2005; Vytrasova *et al.*, 2002**). In addition to the economic losses associated with bakery products, another concern is the possibility of mycotoxin production (**Hocking, 1988**).Therefore, the presence of toxinogenic fungi and mycotoxins in food and grains stored for long periods of time present a potential hazard to human and animal health. Effectively, the use of synthetic chemicals to control food deterioration has been restricted because of their carcinogenicity, teratogenicity, high and acute residual toxicity, and other effects on food and humans (**Tripathy and Dubey, 2004**). Resistance to chemical fungicides also has become a significant problem, and the need for the development of new safe and biodegradable alternatives has increased (**Mourey and Canillac, 2002**). Considerable interest has developed on the food preservation by the use of essential oils to effectively retard growth and mycotoxin production (**Skandamis *et al.*, 2001**). The use of natural plants extracts provides an opportunity to avoid chemical preservation, thus the search for new antifungal material from natural sources for food preservation has increased (**Soliman and Badea, 2002; Irkin and Korukluoglu, 2007**). More than 1340 plants are known to be potential sources of antimicrobial compounds but only few have been studied scientifically (**Wilkins and Board, 1989**). Essential oils and extracts obtained from many plants have recently gained popularity and scientific interest (**Tepe *et al.*, 2005**), because of their antibacterial properties (**Mourey and Canillac, 2002**), EOs or their components which have exibited antiviral (**Bishop, 1995**), antimycotic (**Mari *et al.*, 2003**), antitoxigenic (**Julgal *et al.*, 2002**), antiparazitic (**Pessoa *et al.*, 2002**) and insecticidal (**Karpouthsis *et al.*, 1998**) properties.

The objective of our study was evaluation of the antifungal activity of 5 EOs by vapor contact against the selected fungal species of the genus *Penicillium* isolated from bakery products and their ability to affecting production of selected mycotoxins.

MATERIAL AND METHODS

Fungal isolates

A total of five isolates isolated from different bakery products were used. Two of them, *Penicillium citrinum* and *P. chrysogenum* were isolated from breadrolls and *P. hordei, P. citreonigrum* and *P. viridicatum* from bread. These isolates belong to the collection of microorganism at the Department of Microbiology of the Slovak Agricultural University in Nitra. They were inoculated on Czapek Yeast Autolysate Agar (CYA) **(Samson *et al.*, 2002)** dishes.

Essential plant oils

The essential oils used in this study were extracts of thyme (*Thymus vulgaris* L.), clove (*Syzygnium aromaticus* L.), basil (*Ocinum basilicu*m L.), jasmine (*Jasminum officinale* L.) and rosemary (*Rosmarinus officinalis* L.). They were all supplied by Calendula company a.s. (Nová Ľubovňa, 238 A, Slovakia). The gas chromatography analyse of main components of each essential oils were determined by Calendula company a.s.. Essential oils were extracted by hydro distillation and its quality and stability were certified by suppliers.

Table 1 The major constituents of essential oils analyzed by Calendula company a. s.

Essential oils	Compound	Amount (%)
Thyme	ρ-cimene	40±3
	thymol	32±2
Clove	eugenol	85±3
Basil	metylchavicol	75±2
Jasmine	–	–
Rosemary	α-pinene	19±1
	campfene	9±1
	β-pinene	5±1
	cineol	25±1
	ρ-cimene	17±1
	campher	19±1
	α-terpineol	2.5±0.2
	bornylacetate	0 – 2.0
	borneol	2.0±0.5

Antifungal activity of essentials oil

The antifungal activity of selected EOs was investigated by microatmosphere method. The following method allows the effect of volatile fraction of the EOs to be studied. The test was performed in sterile Petri dishes (Ø 90 mm) containing 15 ml of CYA. Isolates of potential toxigenic fungi were used for toxigenic analyse by thin layer chromatography (TLC) method adapted from **Samson *et al*** (**2002**), modified by **Labuda and Tančinová** (**2006**).

Microatmosphere method

Evaluation by filter paper was made by the method adapted from **Guynot *et al*.** (**2003**). Dishes were kept in an inverted position. A sterilized filter paper (square of 1 x1 cm) was placed in the center of the lid and 50 µl of pure EOs (100/0; v/v; oil/diluent) were added on it. Filter paper disks impregnated with DMSO (50 µl) were only used as control to confirm no solvent effect of bioactivity. Each fungus was inoculated in the center on Petri dishes with needle – inoculated. Dishes were tightly sealed with parafilm and incubated for fourteen days at 25 ± 1 °C (three replicates were used for each treatment). Diameters (Ø mm) of the growing colonies were measured at the 3rd, 7th, 11th and 14th day with a ruler. The essential oils able to inhibit each fungus (visible inhibition – non growth of fungus) were used in the following test.

Minimum fungicidal concentration (MFC)

The minimum fungicidal concentration (MFC) of the essential oils with the most significant activity was determined by method of graded concentration of oils. The essential oils dissolved in dimethyl sulfoxide (DMSO) were prepared at concentration of 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80 and 10/90 (v/v; oil/diluent). Cultivation was carried out the same way as before (at 25 ± 1 °C for 14 days, measured at the 3rd, 7th, 11th and 14th day).The test were performed in triplicate. The MFC was regarded as the lowest concentration of the oil that did not permit any visible growth in comparison with control sets. Data from both methods were evaluated statistically by 95.0 % Tukey HSD test.

Mycotoxins screening by modified agar plug method

After microatmosphere method of 14 days of cultivation with EOs (antifungal activity of 100 % of each tested essential oils with a control sets) three isolates, concretely *P. chrysogenum* and *P. hordei* were tested for a production of roquefortine C (RC) and *P. citrinum* for citrinin (C) by TLC method. Three small pieces (each 5x5 mm) were cut from the colony growing on CYA and placed into 1.5 ml Eppendorf vials. Then 500 µl of extraction solvent (chloroform:methanol, 2:1, v/v) was added to vials containing the agar plugs and shaken on a vortex for at least 2 minutes. Extracts (30 – 50 µl) were applied afterwards as spots to the TLC plate (Silicagel 60, Merck, Germany) 1 cm apart. Consequently the spots were dried and plates were developed in a toluene:ethylacetate:formic acid (6:3:1, v/v/v) solvent system that gave an average R_f value of 0 – 0.68 for citrinin and 0,19 for roquefortin C. Mycotoxin visualization of citrinin was directly detectable as a coloured spot (yellowgreen tail) under UV – light (365 nm). Roquefortin C (RC) was visualized by spraying $Ce(SO)_4$ and after drying detected as an orange spot in daylight. Mycotoxins standards were obtained from Sigma – Aldrich (Germany).

RESULTS AND DISCUSSION

Recently, the scientific interest in biological properties of EO has been increased. New researches about biological active secondary compounds present in EO of plants have been seen as a potential way to control fungal contamination (**Burt, 2004; Soliman and Badea, 2002; Tajkarimi *et al*., 2010**). In this study, we evaluated the ability of 5 essential oils to inhibit bakery spoilage fungi – species of genus *Penicillium*. The major components of EO are listed in Table 1.
The aim of our study was to find the activity of the volatile components of clove, basil, jasmine, thyme and rosemary essential oils against fungal growth and mycotoxins production of *P. citrinum, P. chrysogenum, P. hordei, P.citroenigrum* and *P. viridicatum.* All of the tested pure EOs which affected the fungal growth are presented in Table 2. The EOs of thyme and clove were determined as the most effective against growth of tested isolates. This antifungal activity is reported in several studies. **Guynot *et al*. (2003)** reported that volatile fraction of 5 tested EOs (cinnamon leaf, clove, bay, lemongrass and thyme) had potential antifungal activity against the more common fungi causing spoilage of bakery products (*Eurotium amstelodami, E. repens, E. rubrum, Aspergillus flavus, A. niger* and *Penicillium corylophilum*) which were tested. This same effect was observed by **Rodríguez *et al*., (2007),** that the clove EO totally inhibited all of tested isolates including two *Penicillium* species (*P. nalgiovense* and *P. roqueforti*). **Lis-Balchin and Deans (1997)** reported that strong antimicrobial activity could by correlate with essential oils containing high percentage of monoterpens, eugenol, cinnamic aldehyde and thymol. Thyme and clove EOs revealed high abundance of eugenol (85 %) and thymol (32 %) as main components (Table 1). **Combrinck *et al*., (2011)** also studied antifungal activity of eighteen EO against fungal growth of 5 pathogens including *P. italicum*. Their result showed that thyme oil proved to be the most effective inhibitor for all tested pathogens at the concentration of 1000 µl . l^{-1} and lower with the exception of a resistant *Penicillium* strains. According to the MFC value comparison was thyme and clove EOs determined as the most effective of all with mean 20 % (20/80; v/v) (Figure 1, Figure 2). In addition, in contrast with other effective EOs, thyme and clove EOs showed almost balanced MFC values among all tested isolates. The most sensitive fungal species to all of tested EOs was *P. viridicatum* following with

Table 2 Antifungal activity of tested essential oils (100%) to *Penicillium* spp.

Isolates	Day	Essential oils (mean colony diameter in mm ± SD)					Control
		Clove	Basil	Jasmine	Thyme	Rosemary	
P. citrinum	**3rd**	$0^{a} \pm 0$	$10.50^{ac} \pm 3.97$	$16.83^{b} \pm 1.04$	$0^{a} \pm 0$	$20.00^{b} \pm 8.66$	$26.00^{b} \pm 11.00$
	7th	$0^{a} \pm 0$	$36.00^{b} \pm 5.29$	$27.50^{b} \pm 0.87$	$0^{a} \pm 0$	$29.50^{b} \pm 9.85$	$38.00^{b} \pm 2.00$
	11th	$0^{a} \pm 0$	$40.50^{b} \pm 10.04$	$30.17^{b} \pm 0.76$	$0^{a} \pm 0$	$39.00^{b} \pm 10.58$	$40.50^{b} \pm 6.50$
	14st	$0^{a} \pm 0$	$45.50^{bd} \pm 11.76$	$36.17^{b} \pm 4.80$	$0^{a} \pm 0$	$42.33^{bd} \pm 6.66$	$53.50^{c} \pm 3.50$
P. chrysogenum	**3rd**	$0^{a} \pm 0$	$29.17^{d} \pm 4.04$	$21.17^{c} \pm 7.23$	$0^{a} \pm 0$	$6.83^{b} \pm 3.18$	$18.50^{c} \pm 0.50$
	7th	$0^{a} \pm 0$	$42.50^{d} \pm 1.32$	$24.00^{c} \pm 3.12$	$0^{a} \pm 0$	$14.33^{b} \pm 4.07$	$30.00^{c} \pm 7.00$
	11th	$0^{a} \pm 0$	$48.67^{d} \pm 3.21$	$27.17^{c} \pm 4.51$	$0^{a} \pm 0$	$18.33^{b} \pm 5.58$	$32.00^{c} \pm 7.00$
	14th	$0^{a} \pm 0$	$53.50^{d} \pm 1.32$	$29.00^{c} \pm 5.77$	$0^{a} \pm 0$	$19.00^{b} \pm 6.38$	$33.00^{c} \pm 7.00$
P. hordei	**3rd**	$0^{a} \pm 0$	$14.50^{c} \pm 0.50$	$21.00^{d} \pm 1.00$	$0^{a} \pm 0$	$9.00^{b} \pm 0.87$	$21.50^{d} \pm 1.50$
	7th	$0^{a} \pm 0$	$34.50^{c} \pm 6.00$	$33.67^{c} \pm 2.52$	$0^{a} \pm 0$	$17.83^{b} \pm 1.26$	$40.50^{c} \pm 7.50$

	11th	$0^{a} \pm 0$	$46.67^{c} \pm 8.81$	$44.67^{c} \pm 7.65$	$0^{a} \pm 0$	$19.17^{b} \pm 1.44$	$48.50^{c} \pm 9.50$
	14th	$0^{a} \pm 0$	$50.33^{b} \pm 14.87$	$45.33^{b} \pm 6.35$	$0^{a} \pm 0$	$20.17^{a} \pm 2.02$	$50.00^{b} \pm 10.00$
P. citreonigrum	**3rd**	$0^{a} \pm 0$	$1.00^{b} \pm 0$	$1.98^{c} \pm 0.06$	$0^{a} \pm 0$	$2.33^{c} \pm 0.58$	$4.50^{d} \pm 0.50$
	7th	$0^{a} \pm 0$	$1.67^{a} \pm 0.29$	$42.17^{c} \pm 0.58$	$0^{a} \pm 0$	$23.83^{b} \pm 13.25$	$29.00^{b} \pm 0$
	11th	$0^{a} \pm 0$	$37.83^{c} \pm 2.02$	$61.83^{e} \pm 1.44$	$0^{a} \pm 0$	$26.67^{b} \pm 2.52$	$46.50^{cd} \pm 1.50$
	14th	$0^{a} \pm 0$	$45.50^{b} \pm 8.26$	$73.67^{d} \pm 2.75$	$0^{a} \pm 0$	$36.67^{b} \pm 0.58$	$55.33^{c} \pm 0.58$
P. viridicatum	**3**	$0^{a} \pm 0$	$0^{a} \pm 0$	$18.50^{c} \pm 0.50$	$0^{a} \pm 0$	$7.00^{b} \pm 1.32$	$90.00^{d} \pm 0$
	7th	$0^{a} \pm 0$	$0^{a} \pm 0$	$20.00^{b} \pm 0$	$0^{a} \pm 0$	$18.67^{b} \pm 2.31$	$90.00^{c} \pm 0$
	11th	$0^{a} \pm 0$	$0^{a} \pm 0$	$22.83^{b} \pm 2.75$	$0^{a} \pm 0$	$20.33^{b} \pm 3.79$	$90.00^{c} \pm 0$
	14th	$0^{a} \pm 0$	$17.17^{b} \pm 3.21$	$26.17^{b} \pm 5.25$	$0^{a} \pm 0$	$22.67^{b} \pm 5.39$	$90.00^{c} \pm 0$

Data in the column followed by different letters are significantly different in 95.0 % Tukey HSD test, $P < 0.05$, *P. – Penicillium*, SD – standart deviation

P. citreonigrum (Table 2). *P. viridicatum* was sensitive to clove and thyme EOs all days of incubation and it showed the most significant sensibility to the basil EO with 100 % concentration but only after 11 days of incubation (Table 2). *P. citreonigrum* was sensitive to clove and thyme EOs all days of the incubation, as well as all of tested isolates, but only this isolate showed better sensibility to basil EO at 100 % concentration after 7 days of incubation. The most resistant isolate was *P. chrysogenum*. It was not inhibited by basil, jasmine and rosemary EOs. On the other hand these EOs, in comparison with control, had positive or stimulating effect on the growth of this isolate after all days of cultivation. Result showed that jasmine EO had no noticeable effect on the growth of *Penicillium* spp., but basil, in comparison with the control, had inhibiting effect on their growth (Table 2). The results of authors **Alves- Silva *et al.*, (2013)** showed that the antifungal activity of bush-basil EO was higher against *Mucor racemosus* and *Penicillium chrysogenum*. The EOs of sweet basil had also been reported to have an antimicrobial effect against a variety of gram positive and gram negative bacteria, yeast and molds such as *Mucor piriformis, Aspergillus ochraceus, Penicillium candidum, P. expansum* (**Deans and Ritchie, 1987; Reuveni *et al.*, 1984**).

In this study, according to antifungal activity of tested EOs, they can be divided into three groups. Thyme and Clove, the most active EOs, which completely inhibited growth of tested *Penicillium* spp. The second group with lower activity is compiled from EOs which were well known for their antimicrobial or antifungal activity as basil and rosemary EOs. And third group is formed by EOs with low activity against tested fungal species. In our case, this group include only jasmine EO, had positive or stimulating effect on the growth of some tested *Penicillium* spp. in comparison with control (Table 2).

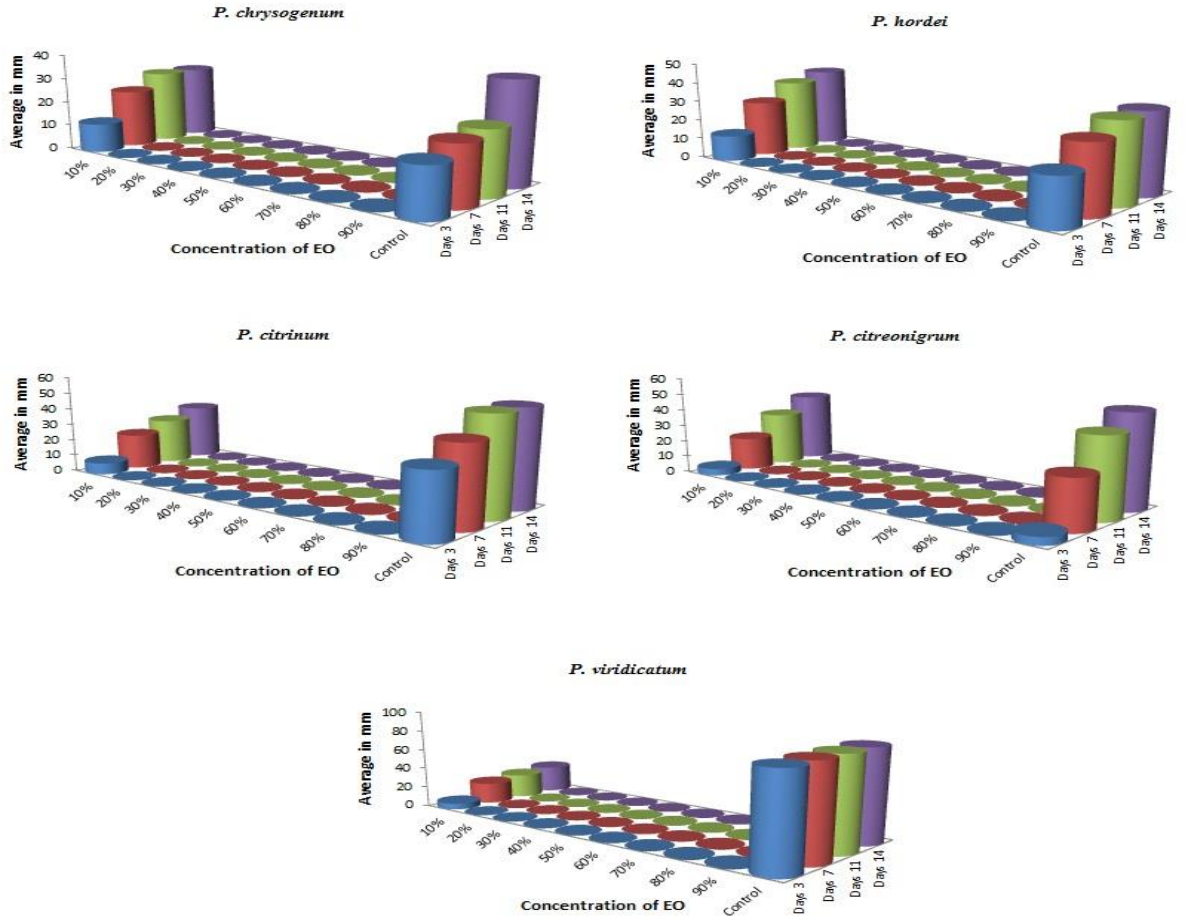

Figure 1 Minimum fungicidal concentration (MFC) of thyme essential oil to *Penicillium* spp.

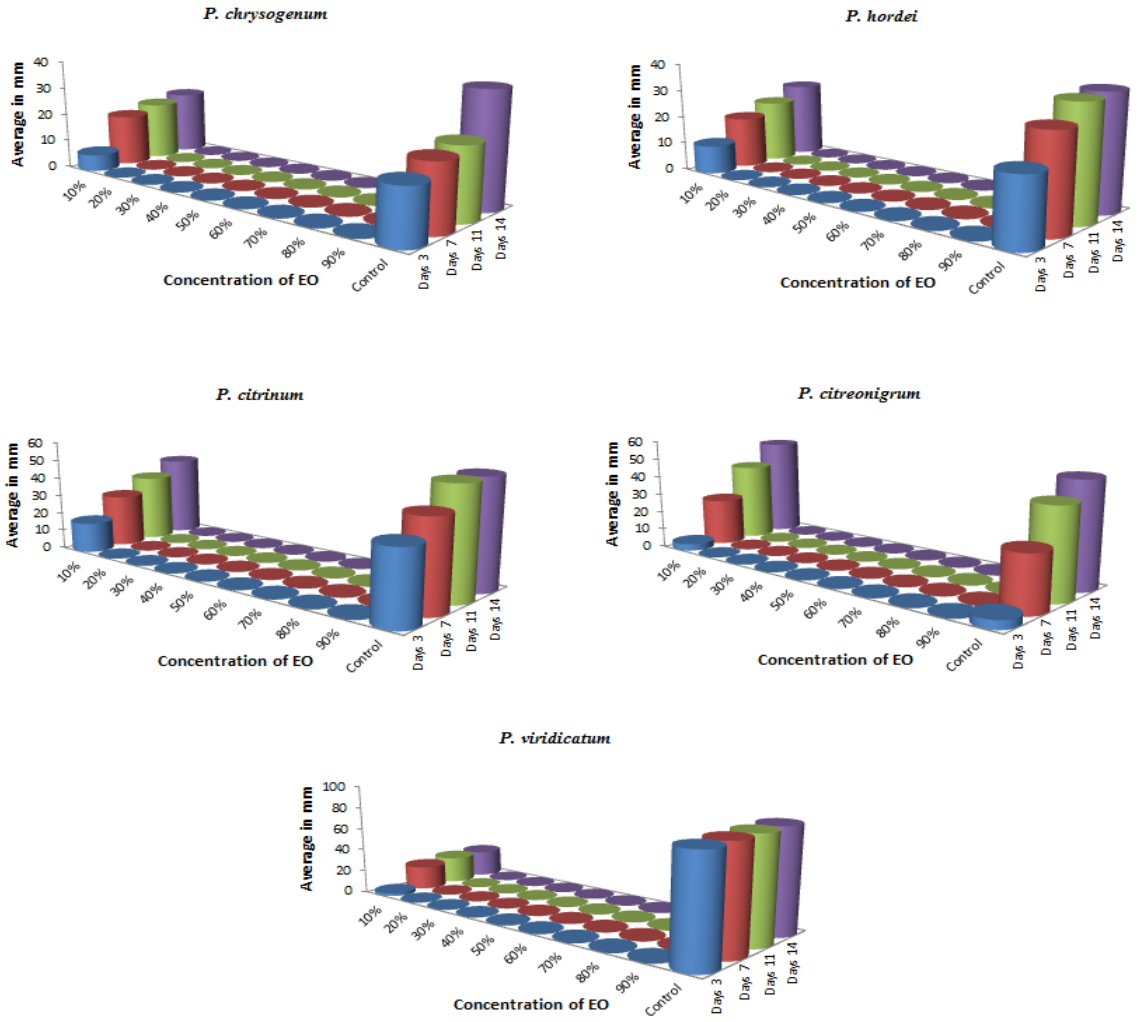

Figure 2 Minimum fungicidal concentration (MFC) of clove essential oil to *Penicillium* spp.

In this study we also tested potentional effect of EOs to affect production of mycotoxins of tested *Penicillium* isolates which are potential toxigenic fungi. After 14 days of incubation with EOs (100 %) with a control sets, *P. chrysogenum* (RC), *P. citrinum* (C) and *P. hordei* (RC) were screened for a production of mycotoxins by TLC chromatography. Our results showed that there was not a significant difference in production of RC and C of control compared with the oil (100 %) treated samples (Table 4). RC and C were not detected only in the samples treated with clove and thyme EOs because the complete inhibited mycelial growth of fungi. In treatments with other tested EOs, all tested mycotoxins were produced, in comparison with control sets. TLC method is a qualitative method and if there is the same difference, it will not be detectable. But there are different results. **Paranagama *et al.* (2003)** studied lemongrass oil ability to inhibit production of alfatoxin. Results obtained from the TLC bioassay and gas chromatography indicated citral A and B as the fungicidal constituents in lemongrass oil and completely inhibited aflatoxins production at concentration 0.1 mg.ml^{-1}. **Velluti *et al.* (2003)** studied inhibitory effect of cinnamon, clove, lemongrass, oregano and palmarosse EOs on growth and fumunosin B_1 production by *Fusarium proliferatum* at different temperature and a_w of samples. They found that cinnamon oregano and plamarose EOs had significant inhibitory effect on FB_1 production at 0.995 a_w of samples and both differenced temperatures, while clove and lemongrass EOs had only significant inhibitory effect at 30 °C. Also **Yamamoto-Ribeiro *et al.* (2013)** tested ability of ginger EO to inhibit production of fumunosin B_1 (FB_1) and B_2 by *Fusarium verticillioides*. Their results showed that the inhibitory effect of FB_1 and FB_2 production was significant at concentration of 4000 and 2000 µl.ml^{-1}.

Table 4 Inhibitory effect of tested essential oils (100 %) on mycotoxins production by *Penicillium* sp.

Fungi	Screened mycotoxins	Essential oils (100%)									
		Thyme		Basil		Clove		Jasmine		Rosemary	
		Rep.	Control	Rep.	Control	Rep.	Control	Rep.	Control	Rep.	Control
P. chrysogenum	RC	$0^{*1}/0^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$	$0^{*1}/0^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$
P. citrinum	C	$0^{*1}/0^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$	$0^{*1}/0^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$
P. hordei	RC	$0^{*1}/0^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$	$0^{*1}/0^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$	$3^1/3^2$	$1^1/1^2$

P. – *Penicillium*, Rep. – repetition, *no screened isolates (no visible growth), 1 – number of screened isolates, 2 – number of positive isolates, RC – roquefortin C, C – citrinin

CONCLUSION

As a conclusion, volatile substances from thyme, basil, clove and rosemary essential oils had a potential antifungal activity against tested *Penicillium* spp. causing spoilage of bakery products. In our study we tested the antifungal activity of EOs against *Penicillium* spp. isolated from bakery products for 14 days with addition of pure EOs (100 %). Results showed that all of the tested EOs had antifungal activity, except jasmine, which appeared to be effective only to isolate *P. citreonigrum* after first 3 days of cultivation. But antifungal activity of tested EOs depends on concentration of EOs and cultivation time. According to all results obtained in our study, we can assume that thyme and clove EOs are the most promising of all tested EOs because of their MFC values which were up to 20 % (20/80; v/v) for all tested isolates. This could become an effective modern alternative without health hazards for customers frequently mentioned in the case

of synthetics fungicide especially for bakery products because they haven't longer shelf life. Therefore, there is not needed a long period vapors effect of EOs. Usually, bakery products are packaged in plastic films after baking and cooling and they are consumed within 1 or 2 months. Therefore, EOs vapors can be good alternative as a protection of bakery products.

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